United States
Environmental Protection
Agency

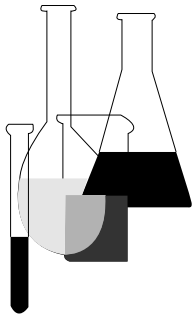
Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–154
April 1996

EPA

Ecological Effects Test Guidelines

OPPTS 850.4200
Seed Germination/Root Elongation Toxicity Test



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

OPPTS 850.4200 Seed germination/root elongation toxicity test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.2750 Seed Germination Root Elongation Toxicity Test.

(b) **Purpose.** This guideline is intended for use in developing data on the acute toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulations. This guideline prescribes test procedures and conditions using seed of commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The EPA will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

(1) *ECX* means the experimentally derived chemical concentration that is calculated to affect X percent of the test criterion.

(2) *Embryo* means the young sporophytic plant before the start of germination.

(3) *Germination* means the resumption of active growth by an embryo. The primary root should attain a length of 5 mm for the seed to be counted as having germinated.

(4) *Hypocotyl* means that portion of the axis of an embryo or seedling situated between the cotyledons (seed leaves) and the radicle.

(5) *Radicle* means that portion of the plant embryo which develops into the primary root.

(6) *Test solution* means the test chemical and the dilution water in which the test chemical is dissolved or suspended.

(d) **Test procedures**—(1) **Summary of the test.** (i) Seed should be separated into appropriate size classes, and that size class containing the most seed should be used exclusively for the test. Fresh test solutions should be added to Petri dishes that have been completely filled with either precleaned quartz sand, 200 μm glass beads, or other inert material. The seed should then be positioned on the substrate allowing adequate room for anticipated growth. It is recommended that the radicle end of the seed be aligned in the direction of this growth. Petri dish lids should be used to hold the seed in place, and the dishes sealed with tape. Deionized or

glass-distilled water should be added to the substrate prior to positioning the seed for those chemicals that are insoluble in water and that should be sorbed to the substrate.

(ii) The dishes should be placed in a seed germinator or other growth facility at a slight angle to facilitate linear root growth. Seed should be incubated in the dark until at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long.

(iii) The number of seed that germinate should be counted, and root lengths measured. Concentration response curves, EC10s, and EC50s for seed germination and root elongation should be determined and reported for each of the species tested.

(2) **Chemical application.** (i) Test chemicals that are soluble in water should be dissolved in deionized or glass-distilled water and added to the substrate in the petri dishes at the start of the test.

(ii) Test chemicals that are insoluble in water but which can be placed in aqueous suspension with a carrier should be suspended in deionized or glass-distilled water with the carrier and then added to the Petri dishes. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers; however, acetone, gum arabic, polyethylene glycol, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Tests of the carrier effect should be included in the test experimental design and conducted simultaneously as controls.

(iii) Water-insoluble chemicals for which no nontoxic water-soluble carrier is available, should be dissolved in an appropriate volatile solvent. The solution and substrate should be placed in a rotary vacuum apparatus, and evaporated, leaving a uniform coating of test chemical on the substrate. A weighed portion of the substrate should be extracted with the same organic solvent and the chemical assayed before the containers are filled. Solvent controls should be included in the experimental design and tested simultaneously. Deionized or glass-distilled water should be added to the treated substrate prior to positioning the seed on the substrate.

(3) **Range-Finding test.** (i) A range-finding test should be conducted to establish if definitive testing is necessary and to determine test solution concentrations for the definitive test.

(ii) The seed should be exposed to a chemical concentration series (e.g., 0.01, 0.1, 1.0, 10, 100, and 1,000 mg/L. The lowest concentration in the series, exclusive of controls, should be at the detection limit of the chemical. The upper concentration, for water soluble compounds, should be the saturation concentration.

(iii) The test consists of one run for each of the recommended plant species or selected alternates. A minimum of 15 seeds per species should be exposed to each chemical concentration and control. The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. The exposure period may be shortened if data suitable to establish the test solution concentration series for the definitive test can be obtained in less time and if the definitive test is to be conducted. No replicates are required and nominal concentrations of the chemical are acceptable unless definitive testing is not required as specified in paragraph (d)(3)(iv) of this guideline.

(iv) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent inhibition of germination or reduction in root growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent inhibition of germination or reduction in growth.

(v) Graphical analysis of the range-finding data facilitates selection of chemical concentrations for the definitive test.

(4) **Definitive test**. (i) The purpose of the definitive test is to determine the concentration-response curves, the EC10s and EC50s for seed germination and root elongation for each species tested, with the minimum amount of testing beyond the range-finding test.

(ii) The seed of each species tested should be exposed to at least 6 concentrations of the chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, and 64 mg/L). The concentration ranges should be selected to determine the concentration response curves between the EC10 and EC50 for both germination and root elongation. Test solutions or substrate extracts should be analyzed to determine chemical concentration prior to use. Selection of seed from the size class lot to be exposed to each test concentration should be unbiased.

(iii) At least three replicates, each with at least 10 seed per species should be tested for each concentration and control.

(iv) Every test should include controls consisting of the same dilution water, conditions, procedures and seed from the same lot used in the exposure group, except that none of the chemical is added. If a carrier (solvent) is needed to suspend or disperse the chemical, a separate carrier control should also be used.

(v) The test period may be ended when at least 65 percent of the control seed have germinated and developed roots that are at least 20 mm long. When both conditions are satisfied, the mean number of seed germinating and mean root length per treatment (and control) can be determined. If the test chemical concentration series does not bracket the EC10 through EC50, for both germination and root elongation, the test should be repeated

(at a higher or lower concentration series). Concentration response curves, EC10s and EC50s for germination and root elongation should be determined for each species tested and reported along with their 95 percent confidence limits.

(vi) Any abnormal seedling development or appearance such as lesions, enhanced root growth (measured), discoloration, swelling, loss of turgor, etc., should also be reported.

(vii) A randomized complete block design is recommended for the definitive test with blocks delineated within the seed germinator or growth chamber. If, for any reason, blocking is not feasible, total randomization within chambers is acceptable.

(viii) Temperature in the germination facility should be recorded hourly. The pH of the test solutions should be recorded at the initiation of the definitive test.

(5) **Analytical measurements—**(i) **Test chemical**. Stock solutions should be diluted with glass-distilled or deionized water to prepare test solutions. Standard analytical methods should be used (if available) to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) **Numerical**. The number of seeds that germinate should be counted and root lengths measured for each definitive test species. All root elongation measurements for a given species should be made sequentially before proceeding to the next species. Root length should be measured from the transition point between the hypocotyl and root to the tip of the root. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration response curves.

(e) **Test conditions—**(1) **Test species**. (i) Test plants recommended for use include:

(A) *Lycopersicon esculentum* (tomato).

(B) *Cucumis sativus* (cucumber).

(C) *Lactuca sativa* (lettuce).

(D) *Glycine max* (soybean).

(E) *Brassica oleracea* (cabbage).

(F) *Avena sativa* (oat).

(G) *Lolium perenne* (perennial ryegrass).

(H) *Allium cepa* (common onion).

(I) *Daucus carota* (carrot).

(J) *Zea mays* (corn).

(ii) Other species of economic or ecological importance to the region of impact may also be appropriate for testing. A minimum of 10 species should be tested.

(iii) Information on seed lot, the seed year or growing season collected, and germination percentage should be provided by the supplier of the seed. Only untreated seed (not treated with fungicides, repellents, etc.) taken from the same lot and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be from the size class which contains the most seed. Damaged seed should be discarded. Standard seed dockage sieves should be used to size seed.

(2) **Facilities—**(i) **Apparatus.** (A) Seed germinator, or other controlled environment chamber capable of maintaining a uniform testing temperature of 25 ± 1 °C is required. In addition, the facilities should include work areas for sizing, counting, and exposing seed for root measurement. If possible, these areas should be isolated from other activities. A fume hood may be needed when testing substances potentially hazardous to human health. Apparatus for distilling and deionizing water are needed unless reagent grade water is used. Refrigeration facilities to hold the seed in cold storage (5 °C) in moisture-proof containers at seed moisture contents of less than 10 percent are also needed.

(B) Disposal facilities should be adequate to accommodate spent glassware, sand, beads, and test solutions at the end of each run and any bench covering, lab clothing, or other contaminated materials.

(ii) **Containers and support media.** A minimum of 210 Petri dishes and sufficient sand or glass beads, or other inert substrate to fill them are needed. Large (200 mm) glass Petri dishes are recommended. Perlite, vermiculite, or native soils should not be used as substrates.

(iii) **Cleaning and sterilization.** (A) All glassware and the substrate should be cleaned before each test following standard good laboratory practice. The substrate should be washed in 7.5 N nitric acid and rinsed with a mild base followed by washes of glass-distilled or deionized water. The pH of the washed substrate should be near neutral. If the glass beads are to be reused, they should be heated to 100 °C for 8 to 12 hours prior to acid washing. A dichromate solution should not be used for cleaning beads or Petri dishes. Sand and plastic Petri dishes should not be reused.

(B) If fungal or other microbial contamination interferes with seed germination so that germination is less than 65 percent in the controls, glassware should be sterilized and/or the seed surface sterilized prior to use, e.g., the seed may be soaked for 10 minutes in a 10 percent sodium hypochlorite solution, then rinsed and soaked for 1 hour in glass-distilled water.

(3) **Test parameters.** Environmental conditions should be controlled to maintain incubation temperature at 25 ± 1 °C in complete darkness. Incubation conditions may have to be adjusted to meet germination and root length criteria in the controls if species other than the 10 recommended for use are tested

(f) **Reporting.** The sponsor should submit all data developed during the test that are suggestive or predictive of phytotoxicity to the Agency. In addition to the general reporting requirements prescribed in 40 CFR part 792, the following should be reported:

(1) Information on the source and history of the seed, germination percentage reported by the supplier, and the seed size class used for testing.

(2) The number of seed of each species per treatment, the number of replicates, carriers, incubation conditions, and seed sterilization procedures.

(3) The concentration of the chemical added to each treatment dish and its pH (pH is optional).

(4) If the range-finding test showed that the highest concentration of the chemical tested (not less than 1,000 mg/L) had no effect on the test species, report the results by species and concentration and a statement that the chemical is of minimum phytotoxic concern.

(5) If the range-finding test showed greater than 50 percent inhibition of germination or root elongation at a test concentration at the analytical detection limit, the results by species and concentration and a statement that the chemical is phytotoxic below the analytical detection limit.

(6) For each species included in the definitive test, means and standard deviations for germination and root length in each treatment. In addition, concentration response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC10s and EC50s identified.

(7) Methods and data records of all chemical and numerical analyses including method validation and reagent blanks.

(8) The data records of the incubation temperature, germination counts, and root length measurements.